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The effect of prolonged storage on the virulence of isolates of *Bacillus anthracis* obtained from environmental and animal sources in the Kars Region of Turkey

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Abstract

The stability of the plasmid mediated virulence factors of *Bacillus anthracis*, a tripartite toxin located on pXO1 and an anti-phagocytic capsule encoded by genes located on pXO2, following long term storage was investigated. A collection of 159 isolates of *B. anthracis* were collected from the Kars region of Turkey between 2000 and 2013 and stored at -20 °C in Brucella broth supplemented with 20% glycerine. 142 isolates were recovered of which one failed to express a capsule upon primary culture. A further 35 isolates yielded a mixture of mucoid and non-mucoid colonies, the majority of which had lost the pXO2 plasmid as determined by PCR analysis. Results would suggest that pXO2 is more unstable than pXO1 and that this instability increases with the length of storage. It is possible that the pXO2 deficient isolates of *B. anthracis* described here could be developed into a vaccine to treat at risk animals in the Kars region as many animal vaccines are based upon pXO2 deficiency.

Introduction

The storage and maintenance of well-defined bacterial isolates is one of the cornerstones of microbiology providing reproducibility and confidence in results. Long term storage can be achieved through several different methods (agar slants, lyophilisation etc.) and has be shown to have an impact on properties of the resurrected cultures (Michel & Garcia, 2003). The maintainance of virulence characteristics is of particular importance for those working in infectious disease modelling or vaccine development. The influence of *in vitro* culture conditions and storage conditions on virulence has been demonstrated in bacterial species such as *Flavobacterium columnare* (Zhang *et al.,*2014) and human pathogens such as *Eshcerichia coli* and *Salmonella* (Huang *et al.*, 2014; Yang *et al.*, 2014).

Bacillus anthracis is a well characterised spore forming bacterium that can establish infections in both animals and humans and was one of the first bacterial species to have an effective attenuated vaccine (Pasteur, 1881; Baillie, 2009). The virulence of *B. anthracis* is mediated by the production of a tripartite toxin and the presence of an antiphagocytic capsule (Green *et al.*, 1985). The genes responsible for these factors are encoded on two seperate plasmids, pXO1 which encodes the toxins and pXO2 which carries the genes for capsule production (Hugh-Jones & Blackburn, 2009).

These plasmids play a key role during infection, however, their contribution to survival outside of an infected host is less clear. Isolates lacking pXO2 have been reported in both environmental samples, as has the spontaneous loss of the plasmid during culture suggesting that under certain conditions pXO2 may be lost (Turnbull *et al.*, 1992). The loss of pXO1 appears to be more infrequent than the loss of pXO2 (Turnbull *et al.*, 1992; Bowen & Quinn, 1999; Pavan & Cairo, 2007) and may account for the routine use of the Sterne strain of *B. anthracis* as a live animal vaccine (Turnbull, 1991).

This current investigation screened the historic *B. anthracis* culture collection maintained at Kafkas University since 2000 to determine the stability of pXO1 and pXO2 in Turkish isolates. In addition to determing the efficacy of the in house storage conditions on strain survival and plasmid stability we also sought to determine if any of the isolates yielded attenuated variants that could potentially be developed into animal vaccines.

Materials and Methods

All culture work and identification of *B. anthracis* was performed in a Class II biosafety cabinet in the Veterinary Faculty of Kafkas University (Kars, Turkey). All consumables and reagents were obtained from either Fisher Scientific (Loughborough, UK) or Sigma Aldrich (Dorset, UK) unless otherwise stated in the text.

Bacterial strains and revival

One hundred and fifty nine *B. anthracis* isolates from the historical collection at Kafkas University were examined at part of this study. Isolates were obtained between 2000 and 2013 from animal and environmental sources using the same cultivation methods by trained personnel. Prior to storage isolates were confirmed as *B. anthracis* by phenotypic assessment (i.e. colony morphology, sensitivity to penicillin G and gamma phage). Isolates were stored at -20^{o}C in Brucella broth (Oxoid, Basingstoke, UK) supplemented with 20% glycerine.

Isolates were revived by the addition of 200 µL defrosted stock to 5 mL Brain Heart infusion broth (BHI; Oxoid, Basingstoke, UK) and then incubated for 48 h at 37^{o}C. After incubation a 0.1 mL sample was spread over the surface of a pre-prepared 7% (v/v) sheep blood agar plate and incubated at 37^{o}C for 18-48 h.

Phenotypic assessment

Individual colonies were assessed using standard microbiological criteria i.e. classical *B. anthracis* colony morphology (ground-glass appearance, flat, opaque, tenacious and grayish-white), sensitivity to penicillin G (10 Units; Oxoid, Basingstoke, UK) and gamma phage

(~10^9 PFU/mL). Once confirmed as *B. anthracis*, isolates were phenotypically assessed for the presence of the pXO1 (toxin production) and pXO2 (capsule expression) virulence plasmids.

The presence of pXO1 was determined using the XO media as described by Hoffmaster & Koehler (1997). In brief, this media contained glucose (0.5% w/v), ferric chloride (40 µg/mL), thiamine hydrochloride (10 µg/mL), glycine (200 µg/mL), L-methionine (40 µg/mL), L-proline (40 µg/mL), L-serine (40 µg/mL), L-threonine (40 µg/mL), $(NH_4)_2SO_4$ (2 mg/mL), KH_2PO_4 (6 mg/mL), K_2HPO_4 (14 mg/mL), sodium citrate (1 mg/mL), $MgSO_4.7H_2O$ (0.005 mg/mL), $MnSO_4.H_2O$ (0.00025 mg/mL), L-glutamic acid (2 mg/mL), and agar (1.5% w/v). Plates were then incubated for 72 h at 37°C and assessed for the presence or absence of growth (Fig. 1a).

The presence of pXO2 was determined on the basis of capsule production. In brief, colonies were streaked to Nutrient Agar plates supplemented with 0.7% (w/v) Sodium bicarbonate and 10% (v/v) Horse serum (Oxoid, Basingstoke, UK; Knisely, 1965). Plates were then incubated for 48 h in candle jars at 37°C. At 24 and 48h post incubation, growth on agar plates was assessed for the production of mucoid (capsule producing) and non-muciod (non-capsule producing) colony types (Fig. 1b). In order to confirm the results obtained using candle jars, capsule production was also assessed by incubation with gradually increasing CO_2 concentration (between 5 and 20%) in a static incubator.

Capsule production was visually confirmed using the McFadyean stain (Owen *et al.*, 2013). In brief, individual colonies were suspended in 10 µL horse serum and smeared over the surface of a microscope slide and allowed to air dry at room temperature. Slides were immersed in absolute ethanol for 10 min and allowed to air dry. A single drop of methylene blue was spread over the slide, left for 1 min and then gently rinsed with clean water and examined under x1000 magnification using a CX21FS1 light microscope (Olympus Corporation, USA) for the presence of capsule (Fig. 1c and d).

PCR assessment of pXO1 and pXO2

Individual colonies of interest (non-mucoid and mucoid phenotypes) taken from bicarbonate agar plateswere used to innoculate 1.5 mL of BHI broth and incubated overnight at 37^{o}C. Control organisms of *B. anthracis* Sterne (pXO1+, pXO2-), *B. anthracis* K-125 (pXO1+, pXO2+) and *Escherichia coli* OP50 were also assessed. Following incubation, overnight cultures were centrifuged at 14,000 x *g* for 10 min, the supernatant discarded and the pellet resuspended in 1.5 mL molecular biology grade water (Eppendorf, Germany). Centrifugation was repeated as before and the pellet resuspended in 100 µL DNase-Rnase free water (Invitrogen, UK). Samples were boiled at 100^{o}C for 10 min in a waterbath, cooled on ice and centrifuged at 14, 000 x *g* for 10 s and stored at -20^{o}C until use.

A summary of primers used in the current investigation can be found in Table 1. A 50 µL reaction mixture (25µL Taq PCR Master Mix Kit (Qiagen, UK), 5µL primer, 5µL Template DNA and 15µL dH_2O (supplied as part of the Taq PCR Master Mix Kit)) of each primer was then run using the following PCR conditions;

For PA5/8, CAP6/103, CapBF/R, CapCF/R and pXO2-007F/R, initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 58°C for 40s and 72°C for 40s with a final elongation step of 72°C for 5 min.

For CapAF/R, initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 40s, 45°C for 40s and 72°C for 40s with a final elongation step of 72°C for 5 min.

Products were analysed on a 1% (w/v) agarose gel stained with SafeView nucleic acid stain (NBS Biologicals, Huntingdon, UK).

Statistical analysis

The degree of association between plasmid profiles and storage time was measured using a chi-square statistic (Preacher, 2001).

Results

A total of 151 (94.9%) isolates were successfully recovered from storage for which 142 (89.3%) were confirmed as *B. anthracis* on the basis of morphology, susceptibility to Gamma phage, penicillin sensitivity and capsule expression.

Confirmed *B. anthracis* isolates originated from animal (n=130) and envionmental samples (n=12). The remaining 17 isolates (10.7%) were shown to be non-viable (n=8) or non *B. anthracis* (n=9).

All isolates identified as *B. anthracis* also grew on XO media suggesting that they contained the pXO1 plasmid (Fig. 1a; Table 2).

When cultured on bicarbonate agar in the presence of CO_2, only one isolate was non-mucoid upon primary culture while a further 35 isolates yield a mixed phenotype of mucoid/ non- mucoid colonies when incubated using a candle jar. Reincubation of these non-mucoid colonies in fixed 5% CO_2 atmosphere failed to restore capsule expression, while 5 of 35 mixed phenotypes regained the ability to express a capsule. Non-muocid colonies from the remaining 30 isolates failed to regain capsule expression suggesting either the complete loss of pXO2 or the inactivation of genes esssential for capsule expression.

PCR analysis was performed on the non-mucoid colonies obtained from all 36 isolates using primers specific for one target on pXO1 (the pag gene) and 5 targets within the pXO2 plasmid, 3 of which are within the capsule synthesis operon. A total of 5 distinct PCR profiles were observed (Fig. 2; Table 3).

Profile A (n=20) comprised of pXO1 primer positive, pXO2 primers negative isolates. Repeated culture (n=3) of these non-muciod isolates in a CO_2 incubator failed to restore capsule expression suggesting that the pXO2 plasmid may have been lost from these isolates.

Profile B (n=10) comprised of pXO1 primer positive isolates which gave a positive response with the Cap6/103 primers but failed to produce a product with the remaining pXO2 specific primers. Following repeated culture (three passages) only one isolate regained the ability to express a capsule.

Pattern C (n=3) comprised isolates which yielded PCR products of the expected size from all primers. All isolates regained the ability to expression a capsule upon repeated culture in the presence of CO_2.

Pattern D (n=1) gave the expected size PCR products with the pXO1 specific and Cap6/103, CapB and Cap C PCR primers but did not regain the ability to express a capsule upon repeated culture (three passages).

The final profile, E (n=1) failed to produce a PCR product with the pXO1 specific primers but produced expected PCR products will all pXO2 specific primers.

The majority (80%) of the strains which produced a mixed capsule morphology were isolated from cattle while the remaining animal isolates came from sheep (20%). The majority (76.6%) of conversions from mucoid to non-mucoid were from strains isolated between 2000 and 2003 while the remainder were from 2005 (3.3%), 2007 (3.3%), 2008 (3.3%), 2011 (3.3%) and 2013 (10%) (Table 2). No significant difference ($P > 0.05$) was observed between the production of a non-mucoid phenotype and sample type. However, conversion to a non-mucoid phenotype was found to increase significantly with increasing length of storage ($P < 0.05$).

Discussion

Although it is well established that *B. anthracis* spores can survive and persist in the environement for decades and still maintain the ability to establish an active infection once a suitable host arises (Wilson & Russell, 1964; De Vos, 1990), there are limited studies which investigate the effect of long term storage on viability and virulence. In this current investigation, 142 (89.3%) isolates of *B. anthracis* were recovered from long term storage. This recovery rate compares favourably to that of Marston and colleagues who were only able to recover 53.8% of isolates following long term storage (Marston *et al.*,2005). The preservation method employed in this study, a liquid medium supplemented with glycerin and the storage at -20°C may account for the greater recovery rate.

The ability of *B. anthracis* to cause anthrax is primarily attributed to the presence of two virulence plasmids; pXO1 which carries the genes which encode the tripartite anthrax toxin (*pagA*, *lef,* and *cya*) and pXO2 which carries the *capBCADE* gene cluster which is responsible for the expression of the poly-D-glutamic acid capsule (Makino *et al.*, 1989; Okinaka *et al.*, 1999ab; Pannucci *et al.,* 2002; Candela & Fouet, 2005). While either plasmid can be lost from the bacteria, it is suggested that pXO2 is more susceptible to loss than pXO1 (Turnbull *et al.*, 1992; Marston *et al.*, 2005).

While the mechanisms responsible for the loss of these plasmids are unclear, factors such as genetic damage, sporulation inadequacies as a result of poor nutrient availability, temperature induced stress and the presence of antibiotics such as novobiocin have all be linked to the phenomenon in the laboratory (Ezzell, 1988; Marston *et al.,* 2005).

A single isolate (PCR profile E) failed to generate a PCR product of the expected size with our pXO1 specific primers it was able to grew on XO media suggesting the presence of at least some or all of the plasmid while all other isolates produced positive results for both PCR and culture assessment. Further studies are required to determine if this failure in PCR assessment was due to the lost of the target DNA or a mutation in the primer recognition sequence.

A single isolate was non-mucoid upon primary isolation and failed to regain capsule expression following repeated culture in a CO_2 incubator. PCR screening with pXO2 specific primers failed to generate any products (profile A) suggesting a complete loss of the plasmid.

A further 35 isolates yielded a mixed phenotype of mucoid/non-mucoid colonies upon primary culture. Of these non-mucoid colonies 20 failed to regain capsule expression following repeated culture on bicarbonate agar in a CO_2 rich atmosphere and yielded negative results with all pXO2 specific primers (profile A) suggesting that the plasmid had been lost.

This study has yielded 21 isolates from the historic collection with the potential to be developed as a live spore animal vaccine due to the non-function or absence of pX02.

The next biggest group (profile B) consisted of 10 isolates of which 9 failed to regain the ability to expression a capsule upon repeated culture in a CO_2 incubator. The fact that one non-mucoid colony producing isolate regained the ability to express a capsule suggests that the conditions within the candle jar may have been non-optimal. It is known that capsule expression is regulated in part by the level of CO_2 in the environment where it is thought to serve as a signal of the mammalian host environment and act as cue for the induction of the virulence factors (Uchida *et al.*, 1993; Drysdale *et al.*, 2005). The constant level of CO_2 achieved by the incubator compared to the candle jar may explain the reactivation of capsule expression in this and other isolates (Fouet, 1996; Green *et al.*, 1985).

PCR analysis of all 10 isolates with pXO2 specific primers, a positive response was obtained with the Cap6/103 primers but failed to yield products with any of the remaining primers. Further studies are required to determine the reasons for this discrepancy and raises the possbility that Cap6/103 may not be specific for pXO2 as first thought.

Isolates that belonged to profile C (n=3) produced mixed mucoid/ non- mucoid colonies upon primary culture in a candle jar but regained capsule expression when cultured in a CO_2 incubator. PCR analysis generated products of the expected size for all of the pXO2 primers suggesting that failure to express a capsule may be due to inefficient regulation of gene expression.

Profile D comprised one non-mucoid isolate which failed to regain the ability to express a capsule when cultured in a CO_2 incubator. PCR primers (Cap6/103, CapB, CapC) specific for some but not all of the pXO2 located targets gave the expected gene products suggesting that the plasmid was present but had been subjected to major mutational events.

The vast majority of isolates which yielded a mixed colony phenotype were intitally isolated betwween 2000 and 2003 suggesting that the length of storage has a significant impact on the stability of pXO2. The results from this study suggest that the storage of *B. anthracis* at -20^{o}C in Brucella broth supplemented with 20% glycerine may not be the optimum approach with which to ensure the maintainence of the orginal properties of the isolate.

Conclusions

The results from this study suggests that the pXO2 plasmid is more susceptible to loss or modification when stored in Brucella broth supplemented with 20% glycerine at –20°C than pXO1 and that this instability increased with the storage duration. While the reasons for this instability have yet to be determined, a number of isolates suggest a complete loss of the pXO2 plasmid. The failure of a small number of isolates to express a capsule was linked to the use of a candle jar suggesting that this is an unreliable method with which to identify capsule producing bacteria. The reasons for the differences in pXO2 primer specificity is currently unclear. Twenty one pXO2 deficient isolates which have the potential to be developed as animal vaccines have been recovered and characterised.

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Table 1. PCR primers used for the confirmation of *B. anthracis* virulence plasmids

Primer	Sequence	Plasmid Target	Location	Product Size (bp)	Reference
PA 8 PA 5	GAGGTAGAAGGATATACGGT TCCTAACACTAACGAAGTCG	pXO1	2452-2471 3048-3029	596	Beyer *et al*. (1995)
CAP 6 CAP 103	TACTGACGAGGAGCAACCGA GGCTCAGTGTAACTCCTAAT	pXO2	506-525 1541-1522	1035	Beyer *et al*. (1995)
CAPA-F CAPA-R	CGATGACGATGGGTGAC AGATTGAAGTACATGCGGATG	pXO2	54942-54931 54266-54287	676	Wang *et al*. (2011)
CAPB-F CAPB-R	GACGAGGAGCAACCGATTAAG AAGAACGCAGGCTTAGATTGG	pXO2	56564-56544 56014-56034	550	Wang *et al*. (2011)
CAPC-F CAPC-R	GTATTAGGAGTTACACTGAGCC GGTAACCCTTGTCTTTGAATTG	pXO2	55554-55533 55208-55229	345	Wang *et al*. (2011)
pXO2-007F pXO2-007R	GCGATGGTGGAACAGGAATG TGCGTTGCTGCCGATATTG	pXO2	4497-4478 3809-3727	688	Wang *et al*. (2011)

Table 2. Summary of phenotypic screening of confirmed *B. anthracis* isolates

Isolate Type	N	Phenotypic characterisation			pXO2 (CO_2 incubator / Candle jar)		
		Penicillin	γ Phage	pXO1	Mucoid	Mixed	Non-mucoid
Cattle	114	114	114	114	89 / 85	24 / 28	1 / 1
Sheep	15	15	15	15	9 / 9	6 / 6	-
Dog	1	1	1	1	1 / -	- / 1	-
Soil	10	10	10	10	10 / 10	-	-
Other	2	2	2	2	2 / 2	-	-

Table 3. Virulence plasmid profiles of *B. anthracis* strains

Sample Type	Strains[a]	Growth on XO medium	Capsule status	PCR results: PA	Cap6/103	CapA	CapB	CapC	pXO2-007
Control	K125 ($pXO1^+$, $pXO2^+$)	+	Mucoid	+	+	+	+	+	+
	Sterne ($pXO1^+$, $pXO2^-$)	+	Non-mucoid	+	-	-	-	-	-
	E.coli OP50 (Neg.cont.)	-	Non-mucoid	-	-	-	-	-	-
Mixed culture- Mucoid	30 (35)	+	Mucoid	+	+	+	+	+	+
Mixed culture- Non-mucoid	Pattern A 20 (20)	+	Non-mucoid	+	-	-	-	-	-
	Pattern B 9 (10)	+	Non-mucoid	+	+	-	-	-	-
	Pattern C - (3)	+	Non-mucoid	+	+	+	+	+	+
	Pattern D 1 (1)	+	Non-mucoid	+	+	-	+	+	-
	Pattern E - (1)	+	Non-Mucoid	-	+	+	+	+	+
Pure culture- Non-mucoid	Pattern A 1 (1)	+	Non-Mucoid	+	-	-	-	-	-

[a]Pattern data in brackets represents samples cultured in candle jars.

Fig. 1. Phenotypic screening of confirmed *B. anthracis* cultures. (a) *B. anthracis* isolate on XO media after 72 h growth. (b) *B. anthracis* isolates on sodium bicarbonate agar after 48 h growth (left side is mucoid and right is non-mucoid colony morphology). (c) no capsule production and (d) capsule production with McFadyean staining. .